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Male populations, which consume soy foods as a major component of their diet, have reduced incidence of prostate cancer. The anti-sex hormone activity in soy has been proposed, but not proven, to provide protection from prostate cancer. Among the soy isoflavones, genistein and daidzein have been found to be the most effective in suppression of breast cancer growth and *in vitro* prostate cancer cell proliferation. We are testing the hypothesis that soy isoflavones, specifically genistein and daidzein, will provide protection from development and progression of prostate cancer in mice lacking functional estrogen receptor-α protein and containing the PBTag transgene (TRAMP mice). We are testing if the phytoestrogens are working through ERα and/or ERβ and other non-ERα estrogen response proteins. The latency of tumor development, tumor size and number, biochemical markers, and histological features will be determined on selected tumors to characterize the progression from normal prostate to neoplastic and metastatic prostate cancer. Through these innovative experiments, we can establish which soy isoflavones are protective against prostate cancer, and we can establish which estrogen receptor pathways are involved.

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Dennis B. Lubahn Dec. 31, 1995

PI - Signature Date

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Introduction

Dietary habits have long been associated with the incidence of chronic diseases, including cancer. However, the vastly complex interactions among the multitude of compounds in food and disease risk have made it very difficult to identify specific chemicals that provide protection. Recently there has been increasing interest in identifying components in foods, which can prevent cancer, so that dietary practices might be specifically modified to reduce cancer risk. In order to achieve this goal, the cellular and molecular mechanisms, through which dietary components function, must be fully defined. The intent of this research is to examine the cellular and molecular mechanisms through which a soy isoflavone extract affects prostate cancer development. Two of the active isoflavone components of soybeans to be specifically tested are the phytoestrogens, genistein and daidzein.

Estrogens are of critical importance in the progression and treatment of prostate cancer. Therefore, factors, which affect the estrogen receptor, are of particular interest for preventative and treatment strategies. There is currently controversy regarding the influence of the soy isoflavones on the estrogen receptor, and hence the ability of these compounds to influence prostate cancer risk. Soy isoflavones have been found to have both estrogenic and antiestrogenic activity in animal models, however the overwhelming data suggest they play a protective role in prostate cancer. The unique and innovative nature of our proposal is the use of the ERα knockout mouse, crossed with the PBTag (TRAMP) mouse, to examine the dependency of the soy isoflavones on ERα to provide protection from sex hormone dependent prostate cancer.

In order to examine the effects on prostate cancer of these two isoflavones, we propose to use soy isoflavone extracts and pure isoflavones, rather than just the complex mixtures of compounds found in whole soy extracts or soy protein (which are commonly used due to their more reasonable availability), to avoid the complications of different amino acid interactions. We also wish to avoid some of the complex interactions that might occur among chemicals in such mixtures, which would limit our ability to interpret the results. Therefore, the diets will be standard casein-based synthetic diets to which isoflavone extracts or pure isoflavones will be added, hence eliminating variables associated with comparisons between casein and soy protein extracts.

The outcome of these experiments will clearly define the dependency of the two isoflavones on the ERα for cancer prevention, thus providing direction for future research. Consumer demand for dietary and lifestyle recommendations, which can reduce cancer risk, is ever increasing. Therefore, rapid advances in identifying potentially protective compounds and their mechanism of action is needed. This highly focused research proposal will open new avenues of investigation, regardless of the outcome of the studies. The expertise of the investigators and their collaborators are an appropriate match to examine these dietary - endocrine interactions. The facilities and support available at the University of Missouri are also fully adequate to perform this work.

The response of normal mouse prostate to estrogen, as well as the absence of response of the ERαKO prostate to estrogen, provides the biological system for examining phytoestrogen responses in TRAMP mice. The ERαKO/TRAMP mouse provides a unique model to identify a mechanism for soy isoflavones in prostate cancer prevention. The ERαKO mice have been thoroughly characterized and a functional colony is well established at the University of Missouri. We are currently in the process of establishing a colony with the ERαKO/TRAMP background. An animal with a null background for ERα function is invaluable for distinguishing biological responses of phytoestrogen dietary components. This initial proposal is intended to focus on answering the question of whether or not the soy isoflavones require a functional ERα to provide protection from prostate cancer in mice carrying the PBTag gene. This is a fundamental question, which can be readily and simply determined with the novel ERαKO/TRAMP mice in that a response to the dietary factors can be clearly distinguished. Whether the data indicate an ERα -dependent or -independent pathway, important new areas of research will be opened, leading to improved dietary recommendations and prevention strategies.

Asian men with a diet high in soy bean products have a low incidence of prostate cancer. However, when Asian men adopt a western diet their incidence of prostate cancer increases. Experimental evidence is lacking to define the specific soy components, which are associated with the low incidence of prostate cancer. It is generally believed the isoflavone components of soy provide the anti-carcinogenic effects, perhaps via their actions through

an estrogen receptor mechanism. To better understand soy's molecular mechanism for protecting against cancer, a diet of soy, or compounds found in soy, will be investigated in the TRAMP (**TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate) mouse model of prostate cancer.

Our initial experiments examine the progression of prostate tumors in a double transgenic mouse that is a cross between the Estrogen Receptor-alpha KnockOut (ERαKO) mouse model and TRAMP mice. Both models are well suited and unique to the study of prostate cancer. The ERαKO mouse is important because of the unknown role that estrogens play in the reduction of prostate tumors, and the TRAMP mouse is important because all male TRAMP mice eventually develop cancer only in the prostate with metastasis similar to that observed in humans. The ERαKO/TRAMP or ER-WT (wild type) /TRAMP mice will be fed a nutritionally adequate, casein-based diet to which an isoflavone extract from soy is added. Other studies have shown that compounds in isoflavone extracts may inhibit mammary carcinoma, and we expect this will be true in the TRAMP model. We plan to examine the isoflavone extract and two soy phytoestrogens, the isoflavones genistein and daidzein. These two soy isoflavones appear to be the most likely to have anti-carcinogenic effects.

It is increasingly evident that risk of developing certain types of cancer can be significantly reduced by dietary intake (Doll and Peto, 1981: Potter and Steinmetz, 1996). The consumption of soyfoods in countries such as Japan and China has been suggested to contribute to the relatively low rates of prostate, colon and breast cancers seen in these populations (Knight and Eden, 1996). This hypothesis is supported by in vitro and animal experimentation. In a review of the literature, Messina et al. (1994) found that 17 of 26 studies reported a protective effect of soy on experimentally induced cancers, whereas none reported that soy increased cancer risk. Potentially anticarcinogenic compounds found in soybeans include the isoflavones, saponins, phytic acid, trypsin inhibitors, phytosterols, soy protein and soy fiber. Among these, the isoflavones have been found in animal and cell culture models to inhibit induced breast tumors (Constantinou et al. 1996), preneoplastic lesions in the colon (Koratkar and Rao, 1997), and prostate cancer cell growth (Kyle et al. 1997; Adlercreutz 1995, 1990).

Two of the primary isoflavones in soy are genistein and daidzein. These isoflavones are phytoestrogens, which are heterocyclic phenols with close structural homology to estrogen. The importance of the isoflavones in providing the anticarcinogenic activity of soy has been suggested by the loss of protection provided by soyfoods devoid of isoflavones (Barnes et al. 1994). Several potential mechanisms through which soy isoflavones may affect cancer have been proposed. Genistein has been found to be an inhibitor of tyrosine kinases (Akiyama et al. 1987). Because of the critical role of tyrosine phosphorylation in the mitogenic regulation of cells by growth factors, genistein may influence tumorigenesis via alteration of cell proliferation. In cultured cells, genistein induced growth arrest, perhaps by blocking tyrosine phosphorylation (Molteni et al. 1995), as well as blocked cell cycle progression (Barnes et al. 1995). Genistein also demonstrated inhibition of angiogenesis (Fotsis et al. 1993), has shown inhibition of mitochondrial aldehyde dehydrogenase (Keung and Vallee 1993) which may limit tumor cell growth, and has acted as a potential antioxidant (Wei et al. 1993) In utero administration of genistein to rats resulted in decreased cell proliferation in mammary glands and enhanced maturation of terminal ducts (Lamartiniere et al. 1995). Genistein has also been shown to stimulate sex-hormone binding globulin (SHBG) production in hepatocarcinoma human liver cancer cells (Adlercreutz 1995; Mousavi and Adlercreutz 1993). Higher levels of SHBGs would decrease levels of free sex hormones such as testosterone and estradiol, thus regulating plasma clearance and uptake of these hormones and decrease the propensity for growth of hormone dependent tumors.

Hence, genistein may have a direct effect as well as an indirect effect on prostate cell differentiation and proliferation. The primary studies on genistein have been in relation to breast cancer, and in numerous experimental animal models, genistein has been found to decrease breast tumor development (Barnes et al. 1990). Peterson and Barney (1991) studied the inhibitory effect of genistein on estrogen receptor negative and estrogen receptor positive human breast cancer cells. Genistein at high concentrations inhibited the growth of both cell lines, independently of their estrogen receptor content. Yet the effects of genistein in reducing prostate tumor development has not been as widely explored. It has also been determined that colon and prostate cancer incidence are significantly higher in Western countries than in Asia (Rose et al. 1986). *In vitro* and *in vivo* studies have been done looking at the effects of genistein in hormone refractory prostate cancer (Naik et al.

1994). Their results show that genistein is inhibitory of growth *in vitro* but fails to stop growth of *Mat-LyLu* cells transplanted into Copenhagen rats (Naik et al. 1994). Genistein has also induced apoptosis in cultured colon tumor cells (Kuo 1996) and soy phytosterols reduced cholic acid-induced hyperplasia in rat colon mucosa (Awad et al. 1997). In the highly metastatic PC3-M prostate cancer cell line, genistein increased focal cell adhesion kinase and cell adhesion (Bergan et al. 1996) and induced apoptosis (Kyle et al. 1997). Adlercreutz (1990) reviewed some of the effects of genistein and phytoestrogens on prostate cancers, listing their potential roles as aromatase inhibitors and tyrosine kinase inhibitors. Specifically, tyrosine kinase activity associated with receptors for epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), platelet derived growth factor (PDGF), insulin, and mononuclear phagocyte growth factor. These in vitro models have yet to define an exact mechanism via which genistein actually affects colon or prostate cancer, however they corroborate the epidemiological association between reduced cancer incidence and populations where soy is a primary component of their diet.

Daidzein has been found to reduce carcinogen-induced mammary tumor incidence and multiplicity in rats (Constantinou et al., 1996). A reduction in DMBA-induced sister chromatid exchange formation was noted in mice fed daidzein or a combination of daidzein and genistein (Giri & LU, 1995). It was proposed that daidzein affects tumor growth in a non-ER (and by implication both a non-ERα and non-ERβ) mediated mechanism, as no effect on estradiol's ability to modulate, either c-fos expression or the development of epithelial metaplasia, was observed in an *in vivo* prostate neoplasia animal model (Makela et al., 1995). A prolongation of prostate tumor development was observed in rats fed a high isoflavone-supplemented soy diet containing both genistein and daidzein (Pollard & Luckert, 1997). Therefore, daidzein's exact mechanism of action on tumor incidence is still unknown.

The effects of estrogens on prostate cancer were recently reviewed by Garnick (1997) and previously by Cox and Crawford (1995). Estrogen therapy, principally the use of DES, is a suggested practice in prostate tumor treatment. Its primary mode of action is through feedback on the anterior pituitary with suppression of gonadotropin secretion and subsequent decrease in testosterone production by Leydig cells of the testis. This would in turn cause a decrease in androgens required for hormone dependent cancer. However, direct effects of DES are also possible. Whether DES works through a classical ERα pathway, or another ER dependent mechanism has not been fully determined. It is possible that genistein, daidzein, and other phytoestrogens work through a similar mechanism. It is important to note that genistein has been shown to bind ERα and ERβ with different specificity, providing potential evidence for an alternative protective mechanism (Kuiper et al. 1997).

The estrogen receptor-alpha knock out (ERαKO) mouse is a unique model in which to test the hypothesis that soy affects prostate cancer development via an estrogen-receptor independent mechanism. The ERαKO mouse was generated using homologous recombination in mouse embryonic stem cells to disrupt the ERα gene (Couse et al. 1995). Estrogen insensitivity is evident in these mice, however male and female sexual differentiation and development occurs (Lubahn et al. 1993). The recently cloned ERβ has been found in the ERαKO mice prostate and other tissues (Couse et al. 1997, Lubahn, Endocrinology, submitted & unpublished data). Other response proteins have not been fully characterized but may mediate specific effects of estrogens in cells. In addition, antiestrogen binding sites (AEBS) have been proposed (Katzenellenbogen et al. 1985). The AEBS were found to be present in equal concentrations in estrogen receptor-positive and -negative breast cancer cells. However, the antiestrogenic and growth inhibitory effects of a variety of antiestrogens correlated better with their affinity for the estrogen receptor than the AEBS, leaving questions about the role of AEBS.

The ERαKO model is ideal for studying the effects of phytoestrogens in an ER minus background. Yet the availability of carcinogens that can be used to target cancer development specifically to the prostate are not available. Several animal models have been developed to look at prostate cancer (Zhang et al. 1997, Pylkkänen et al. 1996, Greenberg et al. 1995, Mäkelä et al. 1995), with most involving injection of a predetermined carcinogen. One model looked at the effects of isoflavones in L-W rats, which are susceptible to spontaneous prostate cancer (Pollard & Luckert 1997), showing a protective effect of these compounds. Yet this model requires MNU at several doses to improve prostate tumor incidence because only 10% of the animals develop

tumor spontaneously. To look at the molecular effects of these isoflavones in prostate carcinomas we are crossing ERαKO mice with a transgenic mouse model, which has been developed (Greenberg et al. 1995) that targets tumor growth specifically to the prostate of male mice. This model, known as TRAMP (Foster et al. 1997) for **TR**ansgenic **A**denocarcinoma of the **M**ouse **P**rostate, was developed by placing the SV40 large T-antigen gene under the control of the rat probasin promoter, which has been shown to be highly and specifically expressed in the mouse prostate (Greenberg et al. 1994) and no other tissues. The probasin (PB) promoter is androgen and zinc regulated, with 2 androgen response elements located in this region. It has been shown to be localized in the ducts and nucleus of prostate epithelial cells, yet the function for PB has yet to be identified. The transgene, known as PBTag, which is highly expressed in the dorsal and ventral prostate lobes, produces in abundance the T-antigen. This oncoprotein is known to bind tumor suppresser products of p53 and Retinoblastoma (Rb). Nearly 100% of TRAMP mice develop prostate cancer spontaneously.

The ERαKO/TRAMP mouse model provides a unique opportunity to examine the role of isoflavones such as genistein and daidzein in prevention of prostate cancer in the absence of classical estrogen receptor. This is a novel and exciting approach to determining a biological response to a dietary component. Regardless of the outcome, the data will open many avenues of research that cannot be fully explored at this time. For example, should we find one or both of the isoflavones to be protective in only the ER-WT/TRAMP mice, future work can be aimed at identifying the estrogen receptor activity of the chemical(s). On the other hand if the isoflavones function independently of the ERα - that is provide protection in both ERαKO and ER-WT mice - future work will be aimed at identifying the cellular events mediated by the compounds. Hence, our experiments will clearly define the route to be examined in future studies.

This study will add new information to the field of diet and cancer relationships. The use of soyfoods, or derivatives of soy with anticarcinogenic activity, is rapidly being developed. Understanding the mechanism through which soy isoflavones affect prostate cancer is crucial to the development of dietary recommendations and clinical applications. Soy is a particularly attractive anti-cancer agent, as it is inexpensive, readily available and can be incorporated into a variety of foods. This project will generate new information regarding the mechanism through which soy affects breast cancer, which can be immediately used by clinicians and basic scientists to further the application of soy as a cancer-preventing dietary component.

In this study we are investigating whether soy isoflavones, specifically genistein and daidzein, will provide protection from development and progression of prostate cancer in mice lacking functional ERα protein and containing the PBTag transgene (TRAMP mice). Phytoestrogens may be working through ERβ or other non-ERα estrogen response proteins. Additionally, selected biochemical and histological markers as described below will characterize progression from normal to neoplastic prostate.

BODY

TECHNICAL OBJECTIVES/EXPECTED RESULTS AND POTENTIAL PROBLEMS: **Specific Aim 1a** is designed to establish the effects of isoflavones in our ERαKO/TRAMP and ER-WT/TRAMP mice. Our experimental groups will be fed diets with or without an isoflavone extract present in the diet. Differences in the latency of tumor development, tumor size and number, and histological features will be examined to assess the protective effect of isoflavones in our model. **Specific Aim 1b** will use diethylstilbestrol (DES) as a control to examine its effects in our ERαKO/TRAMP and ER-WT/TRAMP models. The use of DES in humans has been observed to decrease prostate tumor size, yet its effects through ERα, ERβ and/or other non-ERα estrogen response protein pathways have yet to be fully characterized. If DES proves effective in reducing tumor growth in our ERαKO/TRAMP animals, it will help identify the possibility that it works, perhaps as do isoflavones, through a non-ERα mediated pathway.

Once the protective effects of isoflavone extracts in the diets of our mouse model have been shown, our next step is to determine the individual effects of specific isoflavones. **Specific Aim 2a** will compare the response of ERαKO/TRAMP and ER-WT/TRAMP mice fed diets with or with out the isoflavone component genistein. The latency of tumor development, tumor size, tumor classification, biochemical markers, and prostate

tissue histology will be examined. **Specific Aim 2b** will be done in parallel looking at ERαKO/TRAMP and ER-WT/TRAMP mice fed diets with or without daidzein using an identical protocol to specific Aim 2A.

Since soy isoflavones extracts are complex mixtures of phytoestrogens, it is possible that both ERα and ERβ pathways (and perhaps other estrogen signaling pathways, see Das et al. (Preprint enclosed.) are being used, it is logical to test a combination of genistein and daidzein to determine if additivity or synergy exists between them. **Specific Aim 3a** will compared responses in ERαKO/TRAMP and ER-WT/TRAMP mice fed a combination diet of genistein and daidzein with results compared to those in specific Aim 1a and specific Aim 2 to determine if their is an additive effect between the two isoflavones. **Specific Aim 3b** will determine a potential synergistic or additive effect in our mouse models that may exist between both genistein & daidzein and DES. These aims are designed to identify the possibility of protection occurring through a combination of different molecular pathways. It will be a comparison between results obtained here and those obtained in specific aims 2a and 2b that will help identify differences in potential mechanistic pathways and allow us the ability to determine our direction in future studies. In all studies it will be important to establish dosing concentrations using small number of animals so that we know the concentration of isoflavones actually reaching the blood circulation.

RATIONALE: The biochemical roles of diethylstilbestrol and dietary soy phytoestrogens in the prevention and progression of prostate cancer will be examined *in vivo*. Biochemical and histological markers will be examined to determine their roles in the progression of prostate cancer from hormone dependence to hormone independence.

EXPERIMENTAL PLAN/METHODS: Isoflavone extract will be obtained from Protein Technologies (St. Louis, MO) and genistein and daidzein will be purchased from Indofine Chemical Co. (Somerville, NJ). Thomas Mawhinney, Director of the University of Missouri Experiment Station Laboratory has validated HPLC protocols to analyze genistein and daidzein using commercially obtained chemicals. We will consult with him for the analysis and quantitation of in vivo levels of genistein and daidzein in a natural soy diet so that we can estimate the phytoestrogen composition of the synthetic diet necessary to obtain "natural" levels of phytoestrogens. In addition, the analytical protocols will be used to measure the blood content of the isoflavones in the animals in order to validate the response. This is essential to the validity of the study, in that a measured blood level must be determined to establish a dose relationship.

The experimental design of the animal experiments will be a 2x2 factorial, with male ERαKO/TRAMP and ER-WT/TRAMP mice, diets with genistein (daidzein) or without genistein (daidzein), as shown in the following table. A total of 80 mice per isoflavone experiment will be used. All mice will be started on the appropriate experimental diet at weaning. Mice will be castrated at 5 weeks and a silastic testosterone implant (Van Steenbrugge et al. 1984) placed to remove any potential difference in testosterone concentrations previously noted in wild type and ERαKO mice which might differentially stimulate prostate growth (Rissman et al. 1997, Eddy et al. 1996). 20 mice per group will allow us to examine 4 time points for tumor progression with 5 mice per time point. Tumor progression will be examined at 10 weeks, 15 weeks, 20 weeks, and 30 weeks. Times may be adjusted as needed to provide distinct intermediate stages of tumor progression. Staggered starting times will be used for mice as they become available from the breeding colony. Wild type and ERαKO mice will be age-matched siblings to control for any strain background differences still present in our 8th generation backcross to C57/BL/6J females to remove any residual 129SVJ remaining from the original 129SVJ embryonic stem cells.

TABLE 1

	ERαKO/TRAMP	WT/TRAMP
Diet plus isoflavone extract, DES, genistein, or daidzein	20 MICE (5 in each group analyzed at 10weeks,15wks,20wks, & 30wks)	20 MICE (5 in each group analyzed at 10weeks,15wks,20wks, & 30wks)
Diet minus isoflavone extract, DES, genistein, or daidzein	20 MICE (5 in each group analyzed at 10weeks,15wks,20wks, & 30wks)	20 MICE (5 in each group analyzed at 10weeks,15wks,20wks, & 30wks)

The experimental diets will be prepared in pelleted form by Diets Inc. (Nutley NJ) and provide adequate nutrient composition for mice (See budget justification for details). We will provide the isoflavones as a freeze-dried product to the diet manufacturer. Genistein and daidzein will be mixed in a commercial preparation in pelleted form to provide uniform distribution of the isoflavones in the mixture and a more palatable diet for the animals, due to the length of the feeding period. Upon receipt of the diets, the isoflavone concentration will be confirmed using published HPLC methods (Wang and Murphy, 1994). Should there be any difficulty with obtaining the diets from the cited manufacturer, we will be able to mix the diets in our facility and feed then in powdered form. Food intake will be monitored throughout the study in order to identify any animals, which are not thriving. In breast cancer pilot studies our laboratory mice fed this genistein diet have been found to thrive.

Genistein and daidzein will be fed to the mice at concentrations similar to those found in the diets of humans consuming soybeans as a regular part of their diet. Soybeans contain 10-28 mg daidzein/g and 19-30 mg genistein/g, but processed soybeans, such as tofu, tempeh and miso contain higher concentrations of these compounds (Anderson and Wolf, 1995). Calculations done by Barnes (1995) estimated that a person consuming 35 g of soybeans/day would consume 50 mg of genistein and daidzein, providing a concentration of 3.3 mmol/liter of blood. Hence, an estimation of 1-5 mmol of isoflavone/l of blood can be expected in humans consuming soyfoods. In order to achieve a similar concentration in mice, we will perform an initial dose-response experiment in a small group of mice fed genistein or daidzein. The mice will be fed a range of concentrations of the isoflavones for 5 days and blood analyzed for genistein or daidzein concentration on days 4 and 5. Those diets, which produce isoflavone concentrations within the desired range, will be used. We know of no published experiments in which mice were fed genistein or daidzein from which to extrapolate the diet concentrations, therefore this preliminary experiment is essential. Feed concentrations for DES will be established based on experimental data in humans (McConnell 1991) and mice exposed to DES neonatally (200ng/g) (Vom Saal et al. 1997). DES diets will be mixed and pelleted similarly to the isoflavone diets. The pellets will be made so that each mouse consumes between 4-5μg DES per day. Although none are expected, care will be taken to ensure that undesirable side effects are not observed from this level of DES.

The mice will be crossed, bred and genotyped by Dr. Lubahn and males homozygous for the ERαKO or ER-WT genotype, and containing the TRAMP background, will be assigned to an experimental treatment group immediately post-weaning. Animals will be housed in groups of 2 mice per cage. The experimental diets will be fed ad libitum. Prostate tumor induction will be made visually and manually under the supervision of Dr. Cyndy Besch-Williford, an experienced veterinarian pathologist. She will also do the histological examinations.

Body weights will be recorded every other day, and mice will be palpated after weighing for evidence of tumors. At termination, animals will be killed by CO_2 exposure and blood will be drawn via heart puncture and serum separated. All prostate tumors will be measured with calipers, excised, weighed (Miller and Cygan, 1994). Animals will be examined for evidence of secondary lesions and metastasis. Portions of the prostate tissue will be immediately frozen in liquid nitrogen and other portions will be fixed in 10% neutral buffered formalin for 24 hours and processed for paraffin embedment. Frozen tissue will be stored at -80°C for future analysis and DNA/RNA isolation. The fixed tumor tissue will be sectioned and stained with hematoxylin and eosin or prepared for immunostaining by methods previously described (Rosenfeld et al. Endo paper). Tumors will be graded according to morphologic criteria established for human prostate cancer (Miller and Cygan, 1994). Serum will be analyzed for isoflavone concentration.

Several potential mechanisms through which soy isoflavones may affect cancer have been proposed. Genistein has been found to be an inhibitor of tyrosine kinases (Akiyama et al. 1987). Because of the critical role of tyrosine phosphorylation in the mitogenic regulation of cells by growth factors, genistein may influence tumorigenesis via alteration of cell proliferation. In cultured cells, genistein induced growth arrest, perhaps by blocking tyrosine phosphorylation (Molteni et al. 1995). In utero administration of genistein to rats resulted in decreased cell proliferation in mammary glands and enhanced maturation of terminal ducts (Lamartiniere et al. 1995). Hence, genistein may have a direct effect on cell differentiation and proliferation, which may alter the progression of tumor growth. We plan to examine changes in cell proliferation in prostate tissue obtained from mice fed the isoflavones using flow cytometry analysis of cell cycle distribution. Acridine orange will be used as a

DNA/RNA marker to identify cells in G0/G1 or S+G2/M as previously described (Thornton and MacDonald 1994). The insulin-like growth factors (IGF-I and II) are critical regulators of cell cycle progression, and activate intracellular tyrosine phosphorylation of the IGF-I receptor and cellular signaling proteins. Hence, changes in cell proliferation mediated by the isoflavones may be associated with changes in IGF receptors in the prostate cells. Therefore, we plan to examine the insulin-like growth factor receptor quantity and gene expression in the prostate tissue and correlate changes with the cell proliferation response. Receptor quantity will be determined by immunocytochemistry using flow cytometry (MacDonald et al 1993) and receptor message will be analyzed by quantitative PCR (Zhang et al., 1997 *in press*, Das et al., 1997 *in press*).

Androgen receptor (AR), estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) will be determined from steroid-binding assays as described previously (Nonneman et al., 1992; Welshons et al., 1990) and from western blotting of SDS-polyacrylamide gels. For full recovery of receptor protein, tissues (or control cells) are homogenized directly in the SDS gel loading buffer and boiled for 2 min immediately. Mouse AR is probed with monoclonal anti-AR antibody MA1-150 or with antibodies provided by Betty Wilson (see letter of collaboration), mouse ERα with monoclonal anti-ERα antibody H-222 or H-226 provided by Geof Greene (see letter), human ERα (used in some controls) (see Greene letter) with monoclonal anti-ERα antibody D75, and mouse ERβ with epitope-specific polyclonal anti-ERβ C- or N-terminal antibody (Affinity Bioreagents). Blocking is obtained with non-immune serum (10%) plus Irish cream (2-5%). Immunoreactive receptor protein is determined with peroxidase linked to the second antibody or with [^{125}I]protein A after second antibody for signal amplification. Scanning of the blot or of an autoradiograph of the blot (Ambis) shows linearity with applied receptor protein within a working range that is established for each receptor. While ligand binding steroid receptors will be used in initial assays to confirm quantitation by Westerns, in most later experiments Westerns will be used to avoid problems with endogenous steroids, phytoestrogens, and cross-measurements of ERα and ERβ.

Various enzyme biochemical markers will be measured and attempts made to correlate them with latency of tumor development, tumor classification, and histological features. Our preliminary data strongly supports the idea of quantitating prostate 11β-HSD (Rosenfeld et al., 1997 and Slight et al., 1993) as a biochemical marker for monitoring progression of prostate carcinoma.

The participation of angiotensin converting enzyme (ACE) and renin-angiotensin systems acting both in an autocrine and paracrine manner appear to modulate the magnitude of hyperplasia in BPH and in patients with prostate carcinoma (Darenkov et al. 1994). We will quantitate mouse prostate ACE and have developed a novel quantitative autoradiographic method utilizing ^{125}I-lisinopril (a well-established ACE inhibitor) to localize ACE in prostatic tissue.

It is well established that 5α-reductase (conversion of testosterone to dihydrotestosterone (DHT) in human prostate appears to be related to the histological differentiation of the tumor (Habib et al. 1985). Substantial declines in 5α-reductase activity, often accompanied by an increase in testosterone/DHT ratio, were the most striking differences seen in most of the cases between malignant and non-malignant tissue. We propose to quantitate 5α-reductase activity by a radiometric assay developed in our laboratory (Kelce and Ganjam, 1988) in prostate tissue from our experimental groups.

Data will be analyzed by ANOVA using a 2x2 factorial design. Differences among means will be determined by Least Squares Means. The estimated tumor incidence in mice with the PBTag transgene (TRAMP mice) is extremely high with distinct pathology by 10 weeks of age (in our hands no pathology was observed at 6 weeks with noticeable metastasis at 12 weeks (Gingrich et al. 1996). It appears that the sites of metastasis resemble those in humans, with common sites occurring in the lymph nodes and lungs, and less common sites having been observed in the kidney, adrenals and bone. According to Gingrich (1996), by 28 weeks of age, 100% of the animals show metastasis to the lungs and/or lymph nodes. Therefore, it is anticipated that the design described above will provide adequate statistical power to detect differences among the groups.

MORE EXPECTED RESULTS / POTENTIAL PROBLEMS:

1. If isoflavones exert their protection via ERα, then ER-WT/TRAMP animals fed isoflavone extract/ genistein/ daidzein/ DES diets should develop less aggressive tumors (or develop them more slowly) while ERαKO/TRAMP animals will develop tumors more quickly. All animals fed control diet should develop tumors more quickly.
2. However, if isoflavones exert their protection through ERβ or non-classical ERα mechanism then ER-WT/TRAMP and ERαKO/TRAMP animals fed isoflavone extract/genistein/daidzein/DES diets will develop tumors at an equal rate equivalent to those fed control diets.
2. Additionally, Naik et al. (1994) showed that at least one isoflavone, genistein, was inhibitory of hormone refractory prostate cancer cells *in vitro*, and ineffective *in vivo* when MAT-LyLu cells were implanted, but the appropriateness of the model is not clear and the serum levels of genistein were not measured. So although unlikely, if the isoflavone extract were to prove ineffective, but the DES diet were to work, we would use a whole soy-based diet.
3. Finally, if this whole soy-based diet also proved ineffective, then tissue and blood samples will certainly be available from DES-induced prostate metaplasia to determine differences, as the tumor progresses, in prostate biochemical markers and differential gene expression through differential DNA methylation (Huang et al., 1997) & mRNA display. This will provide valuable information about the roles of various *in vivo* estrogens in inducing prostate-specific genes.

Original Proposed Tasks Time Frame

YEAR 1

Task 1: Breed male mice, and characterize offspring as ERαKO or ER-WT with TRAMP background.

PROGRESS:

This has been accomplished but we have been slowed by breeding difficulties, the need to establish a normal base line, (See Table 2 for baseline analyses), and a recent pinworm infection which must be treated by diet with pinworm medication, which confounds the experimental design (see above). We are now capable (once the pinworm infection has been eliminated) of producing the animals needed for this study.

Task 2: **Specific Aim 1** Examine the tumor formation in ER-WT/TRAMP and ERαKO/TRAMP mice fed casein based-diets with or without a soy isoflavone extract supplement (and concurrently with or without DES)

A) Begin feeding experimental diets at weaning.

B) Perform castration and silastic testosterone implants in mice at 5 weeks of age.

C) Monitor biweekly for tumor formation.

E) Terminate and characterize tumors and biochemical markers at 10, 15, 20, and 30 weeks of age.

F) Determine in isoflavone extract diet mice the genistein and daidzein concentration in blood samples.

PROGRESS:

These experiments have or are about to begin.

Task 3: Determine dietary concentrations of genistein and daidzein to obtain desired blood concentrations

A) Dose-response curve for 5 days in small groups of mice.

B) Analyze blood concentrations of genistein and daidzein on days 4 and 5 by HPLC.

Task 4: Acquire the experimental diets containing genistein or no genistein (and daidzein or no daidzein) from a commercial manufacturer (month 3).

A) Deliver genistein and daidzein to the manufacturer.

B) Obtain the diets.

C) Determine genistein and daidzein concentrations by HPLC and gas chromatography in both diets.

Task 5: **Specific Aim 2** Examine the tumor formation in ER-WT/TRAMP and ERαKO/TRAMP mice fed diets with or without genistein (and concurrently daidzein or no daidzein) (months 12-20) (160 mice total).

A) Begin feeding experimental diets at weaning.

B) Perform castration and silastic testosterone implants in mice at 5 weeks of age.

C) Monitor biweekly for tumor formation.

E) Terminate and characterize tumors and biochemical markers at 10, 15, 20, and 30 weeks of age.

F) Determine genistein concentration in blood samples.

Task 6: Analyze the data and write reports (month 21).

YEAR 2

Task 7: Breed male mice, and characterize offspring as ERαKO/TRAMP or ER-WT/TRAMP (months 18-21).

Task 8: Acquire the experimental diets with or without daidzein/genistein and (daidzein/genistein and DES) from a commercial manufacturer (month 15).

A) Deliver daidzein/genistein and DES to the manufacturer.

B) Obtain the diets.

C) Measure the daidzein concentration by HPLC.

Task 9: **Specific Aim 3** Examine the tumor formation in ER-WT/TRAMP and ERαKO/TRAMP mice fed diets with or without combined daidzein/genistein and (concurrently combined daidzein/genistein and DES) (months 21-29) (160 mice).

A) Begin feeding experimental diets at weaning.

B) Perform castration and silastic testosterone implants in mice at 5 weeks of age.

C) Monitor biweekly for tumor formation.

E) Terminate and characterize tumors and biochemical markers at 10, 15, 20, and 30 weeks of age.

F) Measure genistein/daidzein concentration in blood samples.

Task 10: Analyze the data and write reports (month 30).

Key Research Accomplishments / Progress on Tasks:

Year 1 Progress Report (1999)

Task 1: Establish Colony Breeder Pairs

- Currently there are 40+ breeder pairs (these mice are on the standard Purina 5008 diet).
- Because of the need to switch to feeding breeder pairs (see Task #2) the specific phytoestrogen diet, more breeder pairs are being established
- Vectorette PCR is currently being employed to identify the location of insertion of the PBTag transgene.
  - A band has been identified and is currently being characterized to identify sequence so that primers can be designed to establish a multiplex PCR reaction that distinguishes mice that are heterozygous for the transgene from those that are homozygous. This will help in the expedition of identifying females that are homozygous for the transgene and that can then be used in the breeding scheme.

Task 2: Tumor Formation and diets

- All diets but the phytoestrogen extract diet have been obtained. This diet is currently being ordered.
- We have examined tumor formation in ER-WT, ERαKO/PBTag +/- fed a standard mouse chow diet. Mice were examined at 2, 3, 4, 5, 6, 7, and 8 months of age to establish a scale by which to gauge tumor progression in this model (See Table 1)

Table 1. Progression of prostate carcinoma in ERαKO/TRAMP mice

	Age				
Genotype	**4 m**	**5 m**	**6 m**	**7 m**	**8 m**
TRAMP/ERαKO	20% HYP 50% PIN 30% WDC	100% WDC	100% WDC	83% WDC 17% PDC	33% WDC 33% MWDC 33% PDC
TRAMP/ER-wt	83% HYP 17% PIN	70% PIN 30% WDC	30% PIN 17% WDC 50% PDC	33% WDC 66% PDC	33% WDC 66% PDC

HYP = hyperplasia
PIN = prostatic intraepithelial neoplasia
WDC = well-differentiated carcinoma
MWDC = moderately-well differentiated carcinoma
PDC = poorly differentiated carcinoma

This rapid onset of cancer with delay in dedifferentiation was unexpected. From previous observations, ERαKO males have slightly elevated androgens (Eddy et al. 1996), which would be expected to result in accelerated onset and progression of cancer because the TRAMP transgene is androgen-responsive. However, Gingerich and associates (Gingrich et al. 1997) reported androgen-independence of prostate cancer in TRAMP/ERα-wt mice that had been castrated in the early post-pubertal period (6-8 weeks of age). Circulating androgen present for a few post-pubertal weeks activated the transgene, but all castrated mice developed poorly differentiated carcinoma by end of study (18-24 weeks of age) as compared to two-thirds of intact controls, implying surprisingly that depressed androgen stimulation was associated with cancer dedifferentiation. We are

in the process of measuring hormone levels and transgene expression in cancer cells in various ages of TRAMP/ERαKO and cohort mice to look for an association between androgen levels and cancer type before considering other possible role(s) of ERα and ERβ. In any case, if alterations in androgen levels are influencing the progression of the tumor, we could castrate the animals at weaning and use testosterone filled silastic implants to normalize the androgen levels between treatment groups

- Initially the project was designed to monitor the tumor formation with mice started on diet at weaning. Because of the potential protective effects of exposure to phytoestrogens neonatally and *in utero,* [Literature published (or fully appreciated after new data) after submission of grant, (Levy et al. 1995), (Constantinou et al. 1998), and (Lamartiniere et al. 1995)] diets are now being administered to breeder pairs and offspring continued on diet until termination.

Task 3: Dietary concentrations needed to establish appropriate blood concentrations

- A dose response study has been completed using genistein and daidzein diets at .25 , .50, and 1g phytoestrogen/kg diet. Using HPLC, the .5 g/kg diet seems to give a close representation phytoestrogen concentrations of that found in oriental diets. We are currently awaiting arrival of the phytoestrogen extract diet so that blood concentrations can be identified and an appropriate dietary mixture prepared. Current literature has given a ballpark estimate of desired amounts.

Task 4: Obtain appropriate diets

- Diets have been obtained and HPLC has been used to establish approximate blood concentrations

Task 5: Examine tumor formation in mice fed genistein and casein control diets.

- One group of weanling mice was started on the genistein (1g genistein/kg diet) and casein control diets. 20 mice are included in the group to date with more being added.
  - Tumor progression will be monitored and analyzed at 12 and 30 weeks of age. The first group is scheduled to be ready within 2 weeks.
- Breeder pairs fed the 0.50 g genistein /kg diet are currently being established. Concurrently, breeder pairs fed the casein control diet are also being established.
- Because of the chance for environmental and genotypic variation that may affect the statistical outcome, breeder pairs are also being established on the .50 g daidzein/kg diet, the DES diet and the phytoestrogen extract diet when it arrives. Mice will then be collected from each diet at approximately the same time so that variation is reduced.
- As mice are killed for tissue collection, serum samples are being collected for subsequent HPLC analysis of phytoestrogen concentrations.

Reportable Outcomes

With the data generated from these studies additional outside funding through NIH funding (1P01 ES 10535-01 pending) has been obtained to study additional botanical treatments to prevent prostate cancer.

Future Tasks/Aims Which Hopefully will Follow the Above Studies

Ultimate Overall Goal: Identify component(s) of soy that is (are) protective against prostate cancer and the mechanisms through which protective effects are exerted.

1. Dose response curves, identifying agonist and antagonist levels of the phytoestrogen necessary to elicit a protective effect. Doses used initially in this grant were chosen to be substantially above the minimum dose. The establishment of dose response curves for both DES and isoflavones will allow us to determine the actual concentrations and their effects.
2. Difference in androgen receptor expression as a mechanism of action for active compound (Repeat for ERα and ERβ) using immunocytochemistry, androgen binding assays, and quantitative RT-PCR to identify differences in mRNA levels.
3. Differences in gene expression
    - Methylation studies for potential activation and inactivation of genes
        - Look at different points of development as well as different exposure periods (i.e., prenatal, neonatal, etc.) using genomic screens (Huang et al. 1997) and candidate genes (ERα & ERβ)
    - Differential display of various genes such as SHBG levels, which allow variations in serum free steroid concentrations
4. Repeat experiments using ERβ knock out animal, which will help better identify which receptor is responsible for interaction and protective effects. Animals may be protected from prostate cancer through a combination of both estrogen receptors (ERα or ERβ, and/or a not yet determined pathway (See Das et al. paper enclosed). Future studies will entail using antagonists and knockout mice for known pathways, as well as differences in effects of unlinked responses.
5. Identify the effects of isoflavone extract, as well as genistein and daidzein, given ***after*** tumor growth is detected rather than ***before*** as done in this proposal (i.e. treatment in advanced prostate cancer versus prevention early as proposed here) to determine if it modulates size, development, and metastasis.

Conclusions:

TRAMP/ERαKO mice developed prostate cancer but the onset and progression of cancer was different from that observed in the TRAMP/ERα–wt mice (See Table 2). **This finding is very important because it provides evidence that, either directly or indirectly, estrogens do have an effect on prostate cancer in our mouse model, as is commonly found in humans.** Well-differentiated carcinoma developed in a third of TRAMP/ERα KO mice at 4 months of age, and was the only cancer type in 5 and 6 month-old mice of this genotype. In contrast, no TRAMP/ERα-wt mice had carcinoma at 4 months of age, a third had well-differentiated carcinoma by 5 months of age, and half of the 6 month-old mice had poorly-differentiated carcinoma. Although TRAMP/ERαKO mouse developed prostatic carcinoma at an earlier age than wt cohorts, dedifferentiation of carcinoma did not occur at the same rate as in TRAMP/ERα-wt mice. This trend continued over time in that poorly differentiated prostatic cancer occurred in half as many TRAMP/ERαKO mice as wt cohorts by 8 months of age. Our numbers are still small but this trend is interesting.

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